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WITNESS my hand this Thirteenth
day of March 1998

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ORIGINAL

PROVISIONAL SPECIFICATION FOR AN INVENTION ENTITLED

Invention Title: TREATMENT AND DIAGNOSIS OF AN INFERTILITY CONDITION

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The invention is described in the following statement :

This invention relates to a diagnostic method for an infertility condition giving rise to reduced ability to have offspring and to a method of treating such a condition.

An inability or reduced ability to have children can be quite devastating. A relatively high proportion of couples fall into this category. In the US, for example, it is said that some 10-15% of couples of reproductive age are unable to have children, whereas in the United Kingdom this is 14%. In the context of this patent an infertility condition is to be understood to relate not only the capacity to conceive but also to miscarriage, spontaneous abortion or other pregnancy related conditions, and includes sub fertility.

In the last 20 years or so some hope has been held out to infertile couples with the development of *in vitro* fertilisation (IVF) techniques. These IVF techniques generally take the form of stimulating the female to superovulate, contacting collected ova with sperm *in vitro* and introducing a fertilised ovum into the uterus. Multiple variations of this general process also exist. Despite considerable research and technical advances in the IVF field the rate of successful pregnancy following IVF treatment is still quite low and is in the order of 15 to 25% per cycle. Undertaking an IVF program often causes great distress, especially when there is no resultant successful pregnancy. This taken together with the high cost of an IVF program makes it desirable that alternative approaches to the problem of infertility are sought. There are also health risks associated with IVF programs which can prove to be lethal.

Medawar originally hypothesised that maternal immune accommodation of the semi-allogeneic conceptus may be facilitated by immunological tolerance to paternal transplantation antigens. This hypothesis lost favour when it was found that pregnancy does not permanently alter the capacity of mice to reject paternal skin grafts (5, 46). However, the concept of transient hyporesponsiveness to paternal MHC antigens (46) is now receiving renewed attention, as a recent study by Tafuri *et al* (31) have provided clear evidence to show that during pregnancy, T-lymphocytes reactive with paternal class 1 MHC become 'anergic', or unable to recognise antigen due to internalisation of T-cell receptors. This anergic state was functionally operative from as early as implantation and lasted until shortly after parturition when lymphocytes regained their reactivity.

Just precisely what is responsible for inducing this tolerance has heretobefore been unclear. Additionally the nature of the tolerance is unclear.

Seminal plasma is conventionally thought to function primarily as a transport and survival medium for spermatozoa traversing the female reproductive tract (21), but

recent studies by the inventors have highlighted a hitherto unappreciated role for this fluid in interacting with maternal cells to induce a cascade of cellular and molecular events.

Ejaculation during coitus provokes a neurophilic infiltrate at the site of semen deposition termed the 'leukocytic cell reaction' in a variety of mammalian species (1). In mice, the cascade of cellular and molecular changes initiated by the introduction of semen into the uterus, in many respects, resembles a classic inflammatory response. Within hours after mating, a striking influx and activation of macrophages, neutrophils, and eosinophils occurs in the endometrial stroma (2-4), in association with upregulated expression of major histocompatibility complex (MHC) class II and CD86 antigens by endometrial dendritic cells, followed by enlargement of draining lymph nodes (5,6). This inflammatory response is transient and fully dissipates by the time of embryo implantation on day 4 of pregnancy (2-4), when leukocytes persisting in the endometrium are predominantly macrophages with an immunosuppressive phenotype (7).

The temporal changes in trafficking and phenotypic behaviour of endometrial leukocytes during the period between mating and implantation are likely to be orchestrated principally by cytokines emanating from steroid hormone regulated epithelial cells lining the endometrial surface and comprising the endometrial glands (8). Of particular importance are GM-CSF and interleukin-(IL)-6, the synthesis of which are upregulated at least 20-fold and 200-fold respectively in estrogen primed epithelial cells following induction by specific proteinaceous factors in seminal plasma (8,9) known to be derived from the seminal vesicle gland (10). Previous studies have implicated the surge in epithelial GM-CSF release as a key mediator in the post-mating inflammatory response since injection of recombinant GM-CSF into the estrous uterus is sufficient to produce cellular changes resembling those seen following natural mating (11). The inventors have found, however, that GM-CSF is not wholly responsible, since only subtle differences are seen in the numbers and activation states of leukocytes infiltrating the endometrium after mating in genetically GM-CSF deficient mice. The activity of GM-CSF is likely to be compensated or augmented by an array of C-X-C and C-C chemokines, the expression of which are transiently upregulated after mating (12), and cytokines synthesised by activated endometrial macrophages including IL-1 and tumor necrosis factor-α (TNF-α)(4).

The present inventors have investigated the nature of the seminal factor which acts to stimulate GM-CSF release from the uterine epithelium. Previous experiments have shown that the increase in uterine GM-CSF content is neither the result of introduction

of GM-CSF contained within the ejaculate, nor a consequence of a neuroendocrine response to cervical stimulation, and is independent both of the presence of sperm in the ejaculate and MHC disparity between the male and female (8). A mechanism involving induction of GM-CSF mRNA synthesis in epithelial cells by proteinaceous factors derived from the seminal vesicle was suggested by experiments showing that seminal vesicle-deficient males did not evoke GM-CSF release or a post-mating inflammation-like response in females, and that trypsin-sensitive, high molecular weight material extracted from the seminal vesicle could upregulate GM-CSF release from uterine epithelial cells in vitro (10).

It has however not been clear that this inflammatory response is related to the induction of hyporesponsiveness by the mother to the conceptus or alternatively whether the inflammatory response has a role in enhancing the immune system to combat the influx of foreign matter such as potential pathogenic bacteria is not clear. Nor is there any indication as to what the trigger for the induction of hyporesponsiveness is or indeed that hyporesponsiveness is mediated by semen.

One known prior art document is United States patent specification 5395825 by Feinberg. This specification discloses a finding that suggests that elevated levels of TGFβ in the female reproductive tract is required for the production of fibronectin, to assist implantation. The half life of TGFβ is only a few minutes and it effect on fibronectin is very short term and the administration of TGFβ in the above method can only be contemplated for a very short period of time.

SUMMARY OF THE INVENTION

The inventors have identified TGF-β1 as a principal uterotrophic agent in seminal plasma. Cytokine produced in the latent form in the seminal vesicle is activated in the female reproductive tract where it acts to induce GM-CSF synthesis in uterine epithelial cells, and thereby initiates the post-coital inflammation-like response. Additionally the inventors have shown that semen can act to induce hyporesponsiveness to male antigens.

The inventors have also made the finding that if a female's uterus is contacted with sperm containing paternal antigens together with TGFβ a paternal antigen specific state of hyporeactivity is generated with the mother not being able to reject a subcutaneous injected tumour bearing the same MHC class I antigens as contained on the priming sperm inoculum.

The significance of this is that it is highly likely that certain infertility conditions will be related to the incapacity to produce tolerance to antigens of the male, as a result of either a lack of TGFβ in the seminal fluid of the male, an incapacity of the female to process the TGFβ from an inactive to an active form or an absence or low levels of paternal antigens in the ejaculate. Some women may not respond to TGFβ and therefore they may need direct application of GM-CSF.

The TGF-β1 content of murine seminal vesicle secretions, like that of human seminal plasma (22), was found to be extraordinarily high and second only to that reported for platelet distillate (23). In mammalian species the TGF-β family comprises at least three closely related polypeptides, TGF-β_1, -β_2 and -β_3 (24), which exhibit 70-80 % sequence homology and share many biological actions. Although we have not quantified TGF-β_2 or -β_3 in seminal vesicle secretions, these isoforms are unlikely to contribute substantially to initiating the post mating inflammatory response since the GM-CSF stimulating activity of vesicular secretions in uterine epithelial cultures was almost totally neutralised by antibodies specific for the TGF-β_1 isoform of the cytokine. The molecular weight of the GM-CSF stimulating activity in seminal vesicle fluid (150-440 kDa) is consistent with that of the latent form of TGF-β_1, a complex of 230-290 kDa which comprises of the mature TGF-β dimer (25 kDa) non-covalently associated with a 75-80 kDa latency associated protein and a 130-190 kDa binding protein (23). Additional members of the TGF-β family complexed with other carrier proteins such as the 250-300 kDa binding protein betaglycan (25) may account for the higher molecular weight activity present in murine seminal vesicle fluid and human seminal plasma (22).

The synthesis of TGF-β as a latent complex is believed to have a stabilising effect (26) and focus its activity at the target site by binding to extracellular matrix (27). Evidence for a uterine mechanism for activation of latent TGF-β was provided by the finding that in contrast to activity in the seminal vesicle, the majority of the TGF-β_1 found in the uterine luminal fluid after mating was in the active form. Plasmin or other proteolytic enzymes derived from uterine cells or the male accessory glands (28, 29, 47) may contribute to the activation of TGF-β after ejaculation.

The proposal that components of the ejaculate can indirectly contribute to pregnancy success is also supported by experiments in accessory gland-deficient mice (36, 37) and the finding that poor pregnancy outcome and dysregulated fetal and/or placental growth after embryo transfer or during first pregnancy in various livestock species (38-40) can be partially ameliorated by prior exposure to semen (41, 42). Likewise, studies in humans now clearly identify lack of exposure to semen due to limited sexual

experience, use of barrier methods of contraception, or in IVF pregnancies with increased risk of implantation failure, spontaneous abortion and pre-eclampsia (43-45).

In a broad form the invention could be said to reside in a method of treating an infertility condition by exposure of a prospective mother to at least one antigen of a prospective father and to TGFβ or an effective derivative or analog thereof before attempted conception to elicit a transient hyporesponsive immune reaction to said at least one antigen to thereby alleviate symptoms of the infertility condition.

Preferably the a mucosal surface of the prospective mother is exposed to the antigen, and more preferably the mucosal surface is the genital mucosal surface, however, it is feasible that exposure at other mucosal surfaces can give rise to the transient hyporesponsive immune reaction required. There are two basic reasons that this might be the case, firstly it is known that tolerance to external antigens can be elicited at mucosal surfaces, thus it is known that women that are exposed to seminal fluid orally show evidence of reduced pre eclampsia effects to MHC antigens of the male partner (48) Thus the exposure could be oral, respiratory, gastrointestinal or genital. For example the surface antigen and TGFβ may be presented as an oral or nasal spray, or as a rectal or vaginal gel. Such a gel might for example be a gel such a used in the vaginal gel sold under the brand name PROSTIN (Upjohn Pty Ltd). Alternatively it might be desired to take the TGFβ and the surface antigen in a form that gives exposure to the small and perhaps large intestines, such as perhaps contained in a gelatin capsule.

Whilst a mucosal exposure may be preferred because it is likely to give rise to a transient hyporesponsive immune reaction, it may also be feasible to provide for another route of exposure. Thus the surface antigen and TGFβ may be injected for systemic contact.

It may be desirable to deliver the TGFβ and the antigen together, for example where the two are combined in a gel, or spray, alternatively, it might be desirable to provide a source of TGFβ at the mucosal surface of interest, which might be the genital tract, and the antigen could subsequently be deposited onto the mucosal surface. It is also not yet clear whether the TGFβ needs to be present at the same time as the antigen is present, although it is believed to be preferable, however, it is proposed that it may be possible to have a delay between the delivery of the TGFβ and the surface antigen. Thus an alternative would be to deposit the antigen first perhaps as an ejaculate and then deliver the TGFβ as a pessary after intercourse.

The nature of the relevant surface antigens is not entirely clear, but will presumably be those that are particularly antigenic and prominent either on the sperm, or on the conceptus. The most likely candidates are MHC antigens, and more preferably MHC I. The most efficient manner of presenting these antigens is in the form that they are naturally present - on any appropriate cell of the intended male parent that expresses them and those cells would include sperm cells and may include leukocytes. The antigens may also be presented in biological fluids such as seminal plasma which is known to carry certain male antigens (49). This use of cells other than sperm cells will be pertinent where the cell count of the prospective father is somewhat low. The use of cells other than sperm cells may be preferred where a non-genital route is used. Alternatively the antigens may be presented in purified or semi-purified form, which may or may not be presented on inert or adjuvant carriers, thus for example it may be presented in the carriers known as ISCOMS. This latter approach however is likely to be more technically complex and expensive. It is additionally possible that the antigens may be encoded within sperm cells in the form of mRNA (or other nucleic acid) and this RNA message is then expressed by maternal genital tract cells. It may be that TGFβ therefore plays a role in taking up mRNA to express the antigens in these genital tract cells.

The level of TGF β may be varied, and will vary depending upon which species is being treated. For humans the level of TGFβ will preferably be greater than 50 ng/ml with a total dose of 150ng/ml and more preferably at a concentration of between 100 and 400ng/ml with a total dose of between 100 to 2000ng. The level of TGFβ in normal male semen is in the order of 200ng/ml. This level can be judged empirically when assessing other animals, and thus for horses or cattle the preferred level is expected to be in the order of 100ng/ml. These levels may vary if the TGFβ is supplied in a slow release depot, perhaps as a patch or as a gel or latent TGFβ complex.

The level of exposure to surface antigens may vary, in a preferred form the exposure will be to the prospective mother's genital tract in the form of the prospective father's ejaculate, and the level of exposure will be determined by the cell count and antigenic density on the surface of such cells. Where cells are administered other than in the above manner, a similar number of cells might be used, however, the most effective manner may be determined empirically. It is though that an exposure of leukocytes in the order of 10^7 - 10^9 cells might be the appropriate level of exposure to a mucosal surface.

The specificity of TGFβ to be co-administered with the male antigens is at present not entirely clear, and because $TGF\beta_1$ is thought to be responsible whereas $TGF\beta_{2,3}$ are

not it is more likely that TGFβ1 is to be used. It will however also be understood that various modification might be made to TGFβ1 or indeed TGFβ2, or TGFβ3 which could be effective in eliciting an effective transient hyporesponsive immune reaction either separately or in combination with another agent. Such modified TGFβ's might include substitution, deletion or addition mutants, and might include peptide fragments, which may or may not be incorporated into another protein to make a recombinant protein. Alternatively other members of the TGFβ superfamily may also be used or used as a starting point to developing an analog of the TGFβ activity, on such member is known as activin.

Where unmodified TGFβ is used it will preferably be administered as TGFβ1. The TGFβ1 may be administered in its active form, however, where the prospective mother is capable of activating TGFβ1 it may also be administered in its precursor form. An alternative "delivery" option would be natural TGFβ such as in the form of platelets. Thus in stead of purified TGFβ a preparation of platelets or other source rich in TGFβ may be used.

The exposure is preferably a multiple exposure. The multiple exposure is preferably performed over a period of at least three months, with the mucosal surface being exposed to TGFβ during each exposure to the prospective father's surface antigens. This period of time could however be somewhat reduced, and it may be possible to achieved improvement with one exposure but as a minimum it is anticipated that exposure would be at least one week before conception is attempted. It may also be preferred that non-barrier contraceptive measures be taken prior to the planed conception where the surface antigens are presented on sperm cells and these are administered to the genital tract so that there is some certainty of a period of exposure to the prospective father's surface antigen before conception. This is particularly the case where the fertility condition is of the type where conception takes place but either miscarriage, spontaneous abortion or pre-eclampsia occurs after conception.

It is also envisage that the administration of TGFβ and the at least one surface antigen may need to continue past the prospective date of conception perhaps for the first 12 weeks of pregnancy

In an alternative form the invention could be said to reside in a method of diagnosing an infertility condition in males by testing the level of TGFβ in seminal fluid.

Greater than 70% of TGF-β_1 in seminal vesicles exists in the latent form. The infertility condition might therefore not be due to a lack of TGFβ in the semen of the male partner but it may be that the female cannot process the inactive form of the TGFβ. The invention could therefore also be said to include the method of exposing inactive form of TGFβ to the genital tract of a female and testing for her capacity to convert the inactive form of TGFβ to active TGFβ. If this is found to be the case, the method of treating the fertility condition will include administration of active TGFβ, or alternatively a compound capable of activating TGFβ can be administered, such as plasmin, so as to increase the level of active TGFβ.

In a preferred form the method of treating infertility will first include the step or diagnosing or testing whether the male has adequate levels of TGFβ or the female has the capacity to activate TGFβ, or alternatively whether anti-sperm antibodies exist.

The use of the present invention may be used in conjunction with IVF treatment, whereby the transient hyporeactive immune response is elicited before transfer of the conceptus or gametes is attempted. It is expected however that where the infertility condition is caused as a result of reduced TGFβ level in semen, or capacity to activate TGFβ, it is likely that the trauma of IVF treatment may not be needed and that a 'natural' conception may be possible in its place.

It will be understood that this invention is not necessarily limited to humans, but may also extend to treatment of other mammals.

Some specific disorders that may benefit from the present invention are now discussed to some degree.

Recurrent miscarriage. It is known that approximately 2 -5 % of couples are involuntarily childless due to recurrent miscarriage. The aetiology of recurrent miscarriage is complex but in the vast majority of cases no chromosomal, hormonal nor anatomical defect can be found in the couples and it is believed that the majority of these unknown recurrent miscarriages are due to an immunological mechanism. A variety of therapies trying to modify the mothers immune response to the semi-allogenic fetus have been trialed with variable success. The main therapeutic approach over the past 20 years has been to inject paternal leucocytes into the mother in the hope that this will 'tolerise' her towards these paternal antigens, thereby preventing rejection of the semi-allogenic conceptus and the resulting miscarriage. This therapy has been of limited

success with a meta-analysis of 15 trials concluding that paternal leucocyte immunisation of the mother increased pregnancy rates by only 8 - 10 % (51)

Coulam & Stern (52) have administered seminal plasma from a pooled donor source to the genital tract of women with recurrent miscarriage and were able to produce a non statistically significant increase in live birth rates (60%v 48 %, p=0.29 n= 86). This study however differs significantly from our proposed therapeutic regime because it involved administration of seminal plasma with no antigen bearing cells from a donor other that the prospective father. It is not surprising that this therapy was not that successful because no paternal antigen was administered and therefore favourable tolerance towards the prospective fathers antigen's would only occur if sperm from the father happened to be in the woman's genital tract at the time of insertion of the donor semen pessary. The improvement in pregnancy rates seen may reflect the 'chance' presence of the husband's sperm at the time of insertion of the donor seminal plasma.

The data supporting the present invention has given encouraging results which would indicate that TGFβ may be a beneficial treatment for recurrent miscarriage because of its potent immune modulating capacity. It is expected that administration of sperm in combination with TGFβ will help produce a 'nurturing' tolerant immune response to a future conceptus containing the same MHC class I antigens. Mouse studies currently being performed by the inventors to date have already shown that intra-uterine administration of sperm in combination with TGFβ can increase both fetal and placental weights when that female is later mated with a male of the same MHC class I haplotype.

Adjunct to IVF treatment. It is currently believed that premenstrual pregnancy wastage produces a significant negative contribution to IVF success rates. One theory behind this increased early pregnancy loss is that IVF is an 'unnatural' process that separates the act of intercourse from conception. This would mean that IVF recipients may not be expose to seminal plasma and its associated antigens early in pregnancy. Several animal studies and human investigations have suggested that exposure of the female genital tract to semen is beneficial to subsequent pregnancy outcome. The inventors are currently conducting our own trial on the benefit of intercourse to IVF success. Early results from this trial indicate that women who are exposed to semen by having intercourse around the time of embryo transfer may have a lower miscarriage rate and therefore higher live birth rate. There may be some benefit derived from giving women exogenous TGFβ in combination with their partners sperm/leucocytes at the time of, prior to or after embryo transfer, especially if their partners seminal plasma TGFβ levels are low or sperm numbers are low.

Anti-sperm antibody therapy. A significant proportion of infertility is due to the presence of anti-sperm antibodies in either the male or female partner (53). Seminal plasma has been shown to suppress the formation of anti-sperm antibodies in the female serum and genital tract secretions of the mouse. The active agent responsible for this suppression of the maternal humoral immune response towards sperm has not yet been identified. It is expected that the present invention may in at least some instances block anti-sperm antibody formation. The relationship between maternal anti-sperm antibody formation in women and their partner's seminal plasma TGFβ concentration will be investigated to confirm this. Current therapies for anti-sperm antibodies are not very successful (eg oral steroids or the prolonged use of barrier contraception) or require expensive assisted reproduction therapy. It may be possible that simple administration of a TGFβ containing pessary following intercourse may abrogate this anti-sperm antibody response and enable pregnancy to ensue.

Pre-eclampsia prophylaxis. Pre-eclampsia is believed to be an immunological disorder due to shallow placentation created by an overly aggressive immune attack on the invasive trophoblast which is predominantly paternally derived. There is good epidemiological evidence showing that repeated exposure of a woman to her partner's antigens through intercourse in the absence of barrier contraception decreases her chances of developing pre-eclampsia in a subsequent pregnancy to that partner (54, 55). We could hypothesise that this observation is brought about by the generation of maternal tolerance towards paternal antigens created by repeated exposure during intercourse which then enables the trophoblast cells to invade the maternal decidua to an adequate depth to avoid the development of pre-eclampsia. Some women have a propensity to develop pre-eclampsia every time they become pregnant. This may be due to inadequate priming levels of TGFβ in their partners semen or an inability to process latent TGFβ into a biological active form.

BRIEF DESCRIPTION OF THE FIGURES

For a better understanding, the invention will now be described with reference to a number of examples.

Figure 1. GM-CSF release from uterine epithelial cells after incubation with fractions of seminal vesicle fractions (acid activated or untreated). Values means of triplicate cultures and the dotted line depicts GM-CSF production by epithelial cells cultured with DMEM-FCS alone (A). The TGFβ1 content of fractions measured as immunoactivity (ELISA) or bioactivity (Mv-1-Lu cell assay) (hatched area) (B).

Figure 2. The effect of neutralising antibodies specific for TGF$\beta_{1,2,3}$ and TGFβ_1 on the induction of GM-CSF release induced by 2% seminal vesicle secretions. Significant differences of $p < 0.05$ (*) were determined by the Mann Whitney U test.

Figure 3. The content of TGFβ1 immunoactivity (total and natural active) in uterine luminal fluids collected from females 1 hour after mating with intact, vasectomised (vas) or seminal vesicle deficient (SV-) males. Significant differences of $p < 0.01$(*) were determined by ANOVA and Mann Whitney U test.

Figure 4. The effect of rTGFβ1 on GM-CSF production by uterine epithelial cell *in vitro*. Mean ± SD of triplicate wells are shown.

Figure 5. The effect of intra-uterine injection of rTGF-β_1 on the GM-CSF content of uterine luminal fluid. Fluids were collected 16h after administration of cytokine, or after natural mating. Symbols are values from individual mice and median values for groups are scored. Numbers in parenthesis indicate the number of animals in each group. Significant differences of $p < 0.05$ (*) and $p < 0.01$ (**) were determined using a one way ANOVA followed by Mann Whitney U test.

Figure 6 human cervical cultures, effect of TGFβ_1 and 10% whole semen on GM-CSF output.

Figure 7 Human endometrial cultures. Effect ofTGFβ_1 and 10% whole ejaculate on GM-CSF output.

Table 1. The effect of intra-uterine injection of rhTGF-b1 on the numbers of endometrial leukocytes reactive with Abs specific for all leukocytes (LCA), macrophages (F4/10), neutrophils (RB-6), and activated macrophages/dendritic cells (Ia), compared with estrus (non mated) mice and naturally mated mice. The number of cells reactive with Abs are expressed as the mean (range) percent positivity. Significant differences of $p < 0.05$ (*) and $p < 0.01$ (**) were determined by Mann Whitney U test.

DETAILED DESCRIPTION OF THE INVENTION

Materials and methods

Cell Lines, Media, Cytokines and Antibodies.

RPMI-1640 and low glucose Dulbecco's modified Eagle' medium (DMEM, GIBCO) were supplemented with 10% fetal calf serum (CSL), 20 mM HEPES pH 7.2, $5x10^{-5}$ M β-mercaptoethanol, 2 mM L-glutamine and antibiotics (RPMI-FCS and DMEM-FCS). FD5/12 cells (14) were cultured in RPMI-FCS and mink lung cells [Mv-1-Lu, CCL-64; American Type Culture Collection (ATCC)] and uterine epithelial cells were cultured in DMEM-FCS. Recombinant human (rh)TGF-β_1 was from R&D Systems, recombinant murine GM-CSF was provided by N. Nicola, The Walter and Eliza Hall Institute for Cancer Research, and recombinant human activin and inhibin were provided by J. Findlay, Prince Henry's Institute for Medical Research. Monoclonal antibodies (mAb) used for immunohistochemistry were anti-CD45 (TIB 122), anti-Mac-1 (CD11b, TIB 128), anti-MHC class II (Ia antigen, TIB 120; all from ATCC), F4/80 (15), and RB6-6C5 (16). Mouse anti-bovine TGF-$\beta_{1,2,3}$ mAb (which neutralizes all three mammalian TGF-β isoforms) was from Genzyme (Cambridge, MA) and chicken anti-bovine TGF-β1 mAb (neutralizes TGF-β1, <2% cross reactivity with TGF-β_2 and -β_3) was from R & D Systems.

Mice and Surgical Procedures. Adult (8-12 week) [Balb/c X C57B1]F1 female mice and [CBA X C57B1]F1 male mice obtained from the University of Adelaide Central Animal House were maintained in a minimal security barrier facility on a 12 hour light / 12 hour dark cycle with food and water available *ad libitum*. Females were synchronised into estrus using the Whitten effect (17) and cycle stage was confirmed by analysis of vaginal smears. For natural mating, females were placed 2 per cage with individual males and the day of sighting of a vaginal plug was nominated as day 1 of pregnancy. Male studs used for collection of accessory gland secretions were all of proven fertility and were rested for one week prior to use. For intra-uterine injections, uterine horns of estrus females were exteriorised through a dorsal midline excision and injected with 0.2 - 40 ng rhTGF-β_1 in 50 ml of RPMI / 0.1% BSA, or vehicle only, prior to sacrifice of mice 16 hours later for assessment of luminal cytokine content or collection of uterine tissue for immunohistochemistry. All surgical procedures were performed under anaesthesia using Avertin [1 mg / ml tribromoethyl alcohol in tertiary amyl alcohol (Sigma) diluted to 2.5% v / v in saline; 15 µl / g body weight injected i.p.].

Collection of Reproductive Tract Fluids. Seminal vesicle secretions were extruded from intact glands and solubilised in 6 M guanidine HCl (1:4 v / v), then desalted into DMEM using 5 ml Sephadex G-25 desalting columns (Pharmacia) before application to epithelial cell cultures. Prostate and coagulating gland secretions were extracted by homogenisation of intact glands in 0.5 ml of PBS / 1% BSA, followed by sedimenation of debris at 5000 g. Uterine luminal fluid was collected 16 h after mating or instillation

of rhTGF-β1 into the uterus by flushing each horn with 500 µl of RPMI-FCS. Debris was sedimented at 2000 g and the supernatant stored at -80 °C prior to cytokine assay. In experiments where uterine TGF-β1 was measured, flushings of the right horn were made with 6 M guanidine HCl / 0.1% BSA, and desalted into PBS / 0.1% BSA prior to cytokine assay. For matings with intact and seminal vesicle deficient males the left horn was flushed with DMEM to enable confirmation that adequate insemination had occurred (> 1 x 10^6 sperm per ml).

Chromatography. Approximately 1 ml of seminal vesicle fluid in 6 M guanidine HCl was applied to a Sephacryl S-400 column (40 cm x 16 mm; Pharmacia) equilibrated in 6 M guanidine HCl / 0.05 M Hepes pH 7.4. Fractions of 1 ml were collected, desalted into DMEM and assayed for GM-CSF-stimulating activity. Before addition to uterine culture or TGF-β assay half of each fraction was acid activated as previously described (18).

Uterine Epithelial Cell Cultures. Uterine epithelial cells were prepared as previously described (19) and plated in 1 ml culture wells (Nunc) at 1-2 x 10^5 cells per ml in 500 µl of DMEM-FCS. After 4 h incubation at 37 °C in 5% CO2 to allow cell adherence, a further 500 µl of desalted seminal vesicle fluid in DMEM-FCS, cytokines in DMEM-FCS, or DMEM-FCS alone, were added. Culture supernatants were collected and replaced with fresh medium at 16 hours, then collected again 24 hours later, at which time adherent cells were quantified as previously described (19). All treatments were performed in duplicate or triplicate.

Cytokines and Cytokine Assays. GM-CSF was assayed using the GM-CSF dependant cell line FD5/12, essentially as previously described (19). Cell proliferation was determined by the addition of Alamar Blue (Alamar Biosciences) for the last 24 h of the assay or by pulsing with 1 µCi of [^{3}H]-thymidine per well for the last 6 h of the assay. The minimal detectable amount of GM-CSF was 1 U / ml (50 U / ml defined as that producing half maximal FD5/12 proliferation). TGF-β bioactivity was measured using Mv-1-Lu cells as previously described (Like and Massague, JBC), except that cell numbers were quantified by the addition of Alamar Blue for the last 24 h of the assay. The minimal detectable amount of TGF-β in this assay was 15 pg / ml. Cytokine bioassays were standardised against recombinant cytokines and the specificity of the assays was confirmed by the use of cytokine specific neutralising antibodies. TGF-β1 immunoactivity was measured in a specific ELISA (R&D Systems) according to the manufacturers instructions.

Immunohistochemistry. Uterine tissue was embedded in OCT Tissue Tek (Miles Scientific) and frozen in isopropanol cooled by liquid N_2 , then stored at -80 °C until use. Six µm semi-serial sections were cut from uteri collected at 1400 h on the day of estrus or day 1 of pregnancy, or from mice injected with rhTGF-β1and fixed in 96% ethanol (4 °C / 10 min). For mAb staining, sections were incubated with mAbs (neat hybridoma supernatant containing 10% normal mouse serum [NMS]) and goat anti-rat-horseradish peroxidase (HRP; Dako, 1:20 in PBS containing 10% NMS) as detailed previously (19). To visualise HRP or endogenous peroxidase (to detect eosinophils), slides were incubated in diaminobenzidine (Sigma)(5 mg/ml in 0.05 M Tris-HCl pH 7.2) plus 0.02% hydrogen peroxide for 10 min at room temperature. After counterstaining in haematoxylin the sections were analysed using a video image analysis package (Video Pro, Faulding Imaging, Adelaide) in which the area of positive staining in the endometrial stroma was expressed as a percentage of total cell staining.

RESULTS

GM-CSF Stimulating Activity and TGF-β_1 Co-purify from Seminal Vesicle Fluid. Sephacryl S-400 chromatography of vesicular secretions identified two proteinaceous moieties with the capacity to stimulate GM-CSF release from uterine epithelial cells *in vitro* (10). Further investigations revealed that the activity of material eluting in the 150–440 kDa range was markedly enhanced by transient acidification, whereas the 660 kDa activity was abolished by acid treatment (Fig. 1a).

Since acid activation is characteristic of the TGF-β family of cytokines, we examined the TGF-β content of crude and sub-fractionated seminal vesicle fluid. High concentrations of TGF-β_1 immunoactivity were detected in seminal vesicle fluid [median (range) 74 (36-104) ng / ml]. The concentration of immunoactivity was elevated 3.2 fold by transient acidification, indicating that > 70% of the TGF-β_1 in the seminal vesicle exists in the latent form. The seminal vesicle gland was a far more potent source of TGF-β_1 than other male accessory glands, containing 15 fold more TGF-β1 per mg tissue than the prostate or coagulating gland. Confirmation that TGF-β_1 mRNA is synthesised in the seminal vesicle gland was obtained by reverse transcription-polymerase chain reaction (data not shown).

The presence of TGF-β_1 in the 150-440 kDa fractions of seminal vesicle fluid was investigated by Mv-1-Lu bioassay and TGF-b1 ELISA. Maximal TGF-β_1 immunoactivity was found to co-elute in the same molecular weight range as the acid activated GM-CSF stimulating activity (Fig. 1b). When the TGF-β content of fractions

was assessed using sensitive Mv-1-Lu cells, bioactivity was clearly detectable in the 200–440 kDa fractions (approximately 50 ng / ml) but not in higher or lower Mr fractions. The specificity of this bioactivity was confirmed by the findings that these fractions did not affect the growth of TGF-β resistant mouse fibroblasts (L-cells), and that anti-TGF-$\beta_{1,2,3}$ mAb neutralised the capacity of vesicular fluid fractions to inhibit Mv-1-Lu cell growth.

Anti-TGF-β1 Neutralizes Seminal GM-CSF Stimulating Activity. To further investigate the identity of the seminal GM-CSF stimulating activity, the effect of anti-TGF-β mAbs on the capacity of dilute seminal vesicle secretions to stimulate GM-CSF production from uterine epithelial cell cultures was determined. The elevated GM-CSF output induced by co-culture with 2% crude seminal vesicle fluid was completely neutralized in the presence of anti-TGF-$\beta_{1,2,3}$ mAb and diminished by >80% in the presence of anti-TGF-β_1 mAb (Fig. 2), confirming the identity of the seminal GM-CSF stimulating factor as TGF-β and indicating that the majority of this activity is associated with cytokine of the TGF-β_1 isoform.

Seminal Vesicle-Derived TGF-β_1 is Deposited in the Uterus Following Mating. To determine whether TGF-β_1 present in the ejaculate is instilled into the uterus at the time of mating, uterine luminal fluids were collected by lavage from the uteri of oestrus mice 1 h following mating and their content of both active and total (active + latent) TGF-β_1 immunoactivity was assessed (Fig 3). The median total TGF-β_1 content of fluids harvested after mating with intact males was 3.3 fold greater than that of fluids from unmated mice. Comparable increases in uterine TGF-β_1 content were observed after mating with vasectomised males.

To determine the contribution of the seminal vesicular secretions to the TGF-β content of the uterine lumen after mating, females were mated with males from which the seminal vesicle glands had been surgically removed. There was no significant difference between the luminal TGF-β_1 content of mice mated with seminal vesicle deficient males and unmated females, confirming that secretions from the seminal vesicle gland provide the major source of TGF-β_1 found in the uterine lumen after mating.

The amount by which the uterine luminal content of TGF-β1 was increased after mating with intact or vasectomised males (median increase = approximately 400 pg, assuming a luminal fluid volume of 100 ml) is consistent with what would be expected given our finding that the median concentration of TGF-β_1 in seminal vesicular fluid is 74 ng / ml

and the estimated volume of vesicular fluid transferred to the female during mating is 10 ml (20), approximately 50% of which would give rise to the copulation plug and 50% of which would be deposited in the uterus. Furthermore, the majority of the TGF-β_1 found in the uterus after mating was found to be biologically active prior to acid activation (58% and 68% in females mated with intact and vasectomised males respectively), indicating that uterine secretions, or factors derived from the other accessory sex glands contributing to the seminal plasma component of the ejaculate, may have the capacity to activate the predominantly latent TGF-β_1 derived from the seminal vesicle.

rTGF-β_1 Stimulates Uterine Epithelial Cell GM-CSF Production in vitro and in vivo. To determine the capacity of TGF-β_1 to stimulate GM-CSF production from epithelial cells in vitro, rhTGF-β_1 was cultured with uterine epithelial cells harvested from estrous mice. In each of six experiments a dose dependant increase in GM-CSF production was seen with maximal increase elicited at a concentration of 5 ng / ml. At concentrations exceeding 5 ng / ml the increase in GM-CSF production declined in a dose dependant manner (Fig. 4). Interestingly, epithelial cells harvested from diestrous mice were considerably less responsive to the GM-CSF eliciting effects of TGF-β_1, producing 50% less GM-CSF on a per cell basis than cells from estrous mice. Furthermore, recombinant human inhibin and activin, which are members of the TGF-β cytokine family known to be present at high concentrations in human and rodent seminal plasma, were found to have negligible GM-CSF stimulating activity in this assay system.

To determine whether TGF-β could elicit an increase in uterine epithelial cell GM-CSF output in vivo, rhTGF-β_1 was injected into uteri of oestrus mice and the GM-CSF content of the uterine fluids harvested 16 h later was determined. A dose dependant increase in uterine luminal fluid GM-CSF content was elicited, with maximal GM-CSF output occurring after the administration of 40 ng rhTGF-β_1.This increase in GM-CSF content was comparable to that observed in uteri of naturally mated mice (Fig. 5).

rTGF-β_1 Elicits an Inflammatory Response in the Endometrium in vivo. To investigate the effect of TGF-β_1 on uterine leukocyte parameters, an immunohistochemical analysis was made of the endometrial leukocyte populations in estrous mice 16 h following intraluminal injection of 20 ng rhTGF-β_1. The numbers of endometrial cells reacting with mAbs specific for all leukocytes (anti-CD45), macrophages (F4/80 and anti-Mac-1), neutrophils (RB6-6C5 and anti-Mac-1) and activated macrophages / dendritic cells (anti-MHC class II), and the numbers of endogenous peroxidase positive

eosinophils, were all increased by administration of TGF-β_1 (Table 1). The extent of infiltration, and the phenotypic and spatial distribution of the leukocytes recruited to the uterus in response to cytokine treatment, particularly the prominent accumulation of macrophages beneath the luminal epithelium and adjacent to glands, were very similar to those seen *in uteri* harvested from mice 16 h after natural mating (Fig. 6).

Semen and hypo-responsiveness to paternal MHC antigens

To test the hypothesis that exposure to semen at mating may be important for the induction of anergy to paternal MHC antigens during pregnancy, we have established a model system employing congenic mice in the Balb series. Balb/k (H-2k) females mated with Balb/c (H-2d) males were challenged with Balb/c tumour cells (JR-5 fibrosarcoma) on day 4 of pregnancy. Balb/k females were sufficiently hypo-responsive to Balb/c MHC antigens following a single mating to an intact Balb/c male to inhibit rejection of Balb/c tumour cells, confirming the finding of Tarfuri *et al. (35).* Interestingly however, tumour cells were found to grow regardless of whether or not a pregnancy ensued. Similar results were obtained when the uterus of females were ligated at the oviductal junction two weeks prior to mating. The effect was predominantly paternal strain-specific, since tumour growth was significantly less in intact or uterine-ligated females mated with C57Blk x CBA (H-2b/k) males (Table 1). Together, these findings show that exposure to the ejaculate is sufficient to induce a state of hyporesponsiveness to paternal MHC class 1 antigens in the female. These results are summarised in Table 2.

Effect of TGFβ on hypo-responsiveness to paternal MHC antigens

The effect of intrauterine exposure to sperm and TGFβ on induction of tolerance to paternal MHC. Balb/k (H-2k) female mice were uterine ligated (to prevent embryo fertilization), and two weeks later were synchronised into estrus using GnRH and the 'Whitten' effect. At 2300 h on the night of estrus, mice were cervically stimulated to induce luteal activity. At 0900 h to 1200 h on the next day, mice were anaesthetised and given injections into the uterine luminal cavity of sperm (5,000,000), TGFβ (10 ng), TGFβ + sperm, or carrier (1% BSA) alone (50 microlitres per each uterine horn). Additional groups of mice received subcutaneous injections of TGFβ or TGFβ + sperm. Sperm was recovered from the epididymis of Balb/c (H-2d) males, and allowed to capacitate at 37°C for 90 min prior to use. TGFβ was recombinant human (R&D Systems). Four days later, mice were given subcutaneous injections of JR-5 (Balb/c H-2d, 100,000 in 100 microlitres) fibrosarcoma cells. Tumor diameter was measured 13 days later. These results are summarised in Table 3.

Table 3

group	n	number with tumor	median tumor diameter
intra uterine:			
TGFβ	4	4	7.0 mm
sperm	4	2	3.0 mm
sperm + TGFβ	5	5	9.5 mm
carrier	5	1	4.0 mm
subcutaneous:			
TGFβ	5	2	6.0 mm
sperm + TGFβ	5	1	4.0 mm

The data show that tolerance was induced by exposure to sperm and TGFβ. Less of an affect was achieved with intrauterine injection of TGFβ only, indicating some non-specific effect. Sperm given intrauterine, and TGFβ or sperm + TGFβ given to a site other than the uterus (skin) had only marginal effect.

Effect of TGFβ1on human reproductive tissue GM-CSF production

It can be seen from the results of Figures 6 and 7 that the effect of TGFβ is not confined to mice and also has an effect on stimulating GM-CSF in *in vitro*cultivated ectocervical, and endometrial cells.

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Dated this 6th day of March 1997

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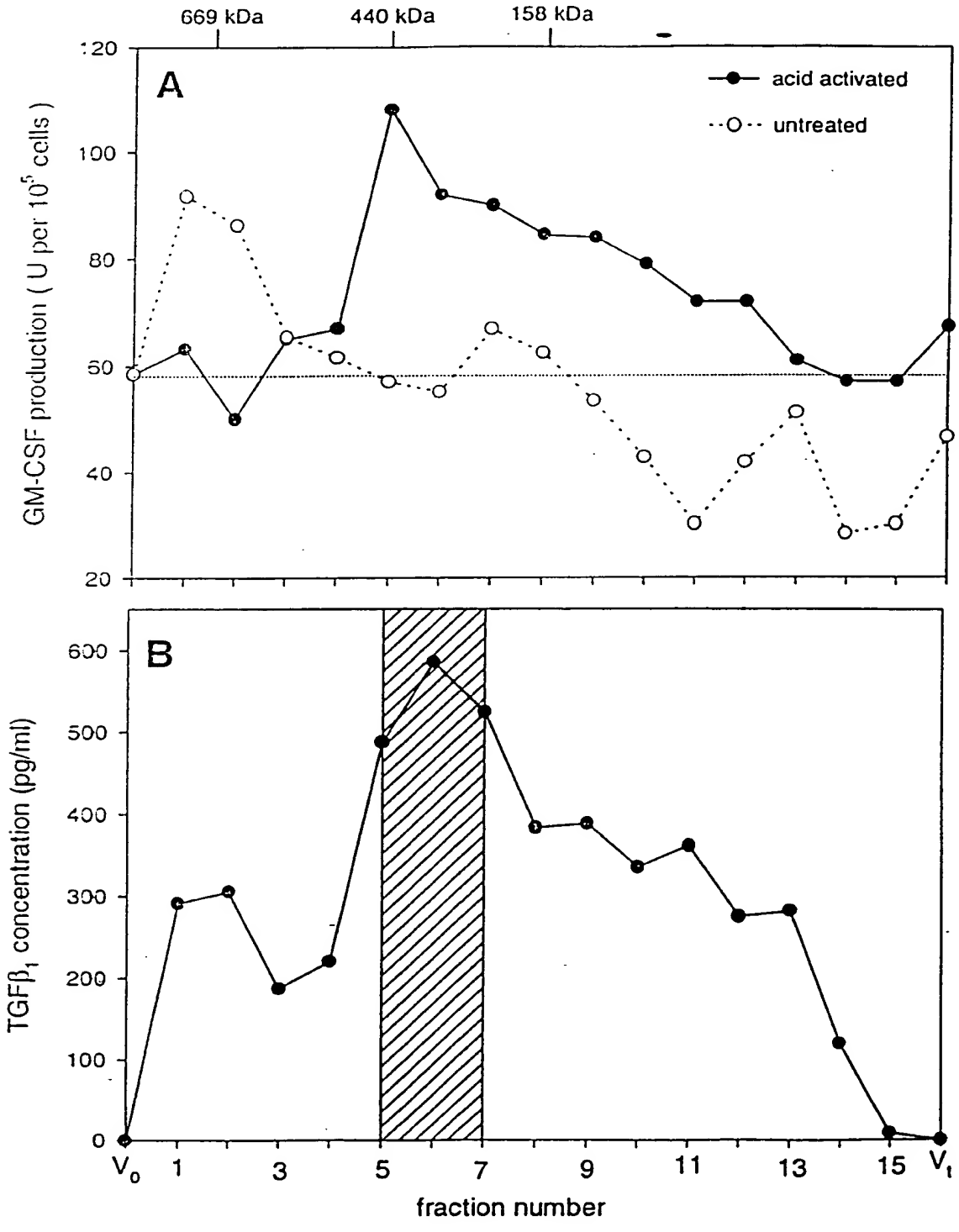
669 kDa
440 kDa
158 kDa
A
acid activated
untreated
GM-CSF production (U per 10^5 cells)
120
100
80
60
40
20
B
$TGF\beta_1$ concentration (pg/ml)
600
500
400
300
200
100
0
V_0
1
3
5
7
9
11
13
15
V_t
fraction number

FIGURE 1

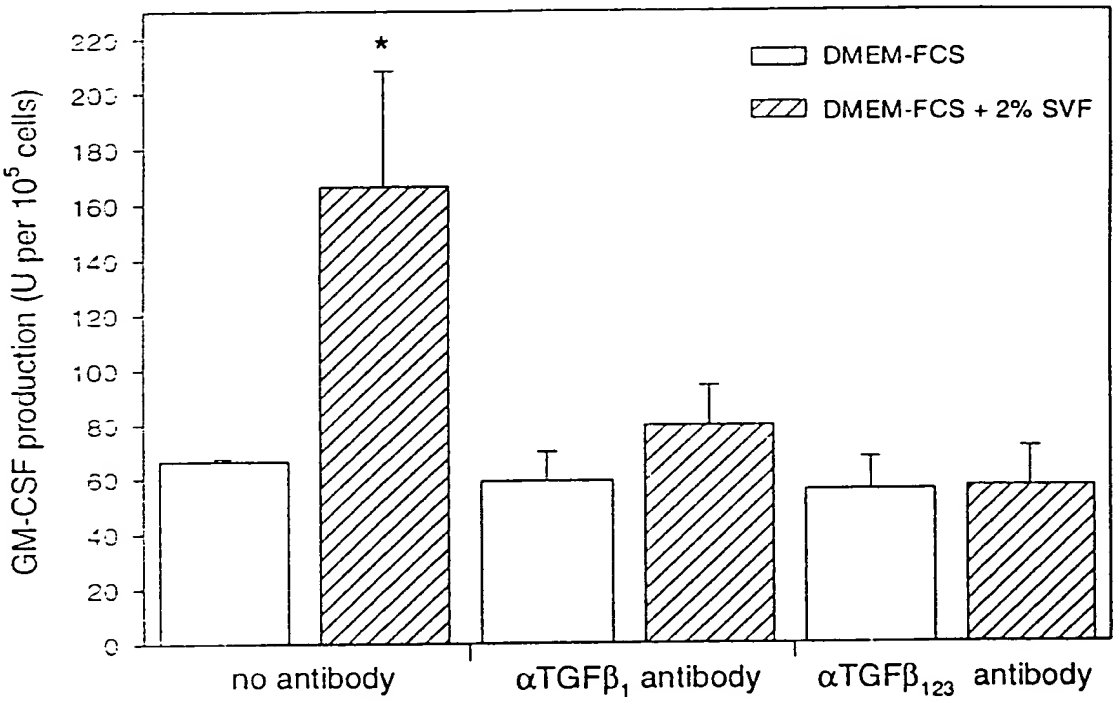
GM-CSF production (U per 10^5 cells)
220
200
180
160
140
120
100
80
60
40
20
0
*
DMEM-FCS
DMEM-FCS + 2% SVF
no antibody
αTGFβ1 antibody
αTGFβ123 antibody

FIGURE 2

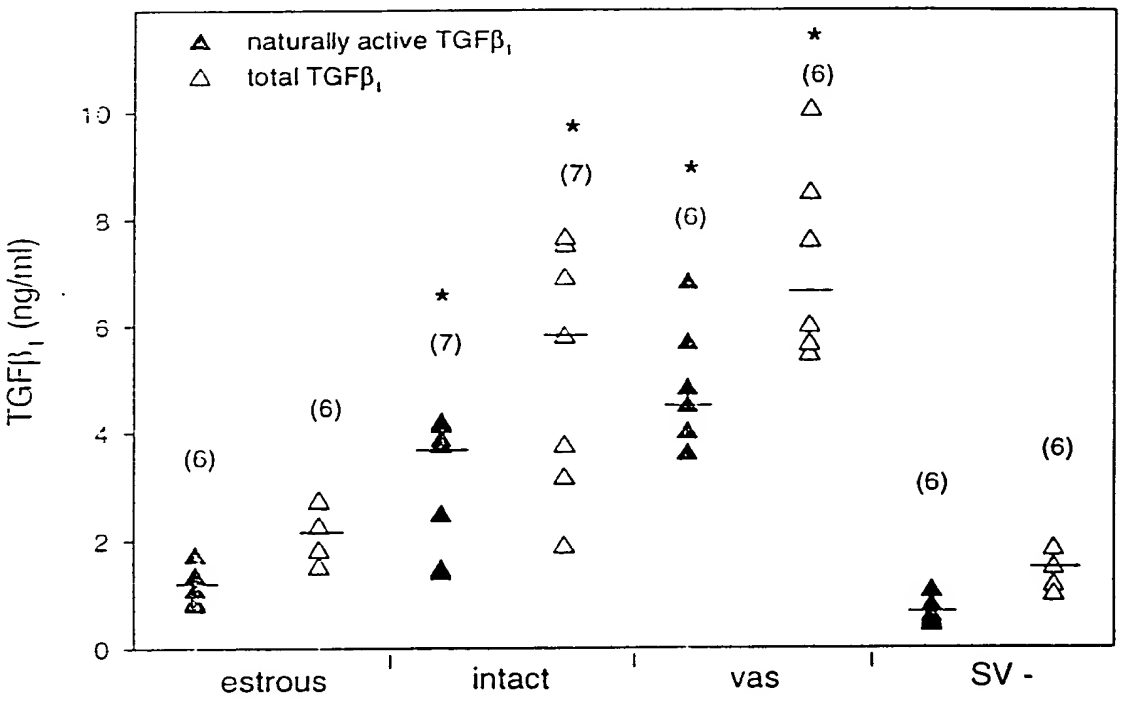
naturally active TGFβ1
total TGFβ1
TGFβ1 (ng/ml)
0
2
4
6
8
10
(6)
(6)
(7)
(7)
(6)
(6)
(6)
*
*
*
estrous
intact
vas
SV -

FIGURE 3

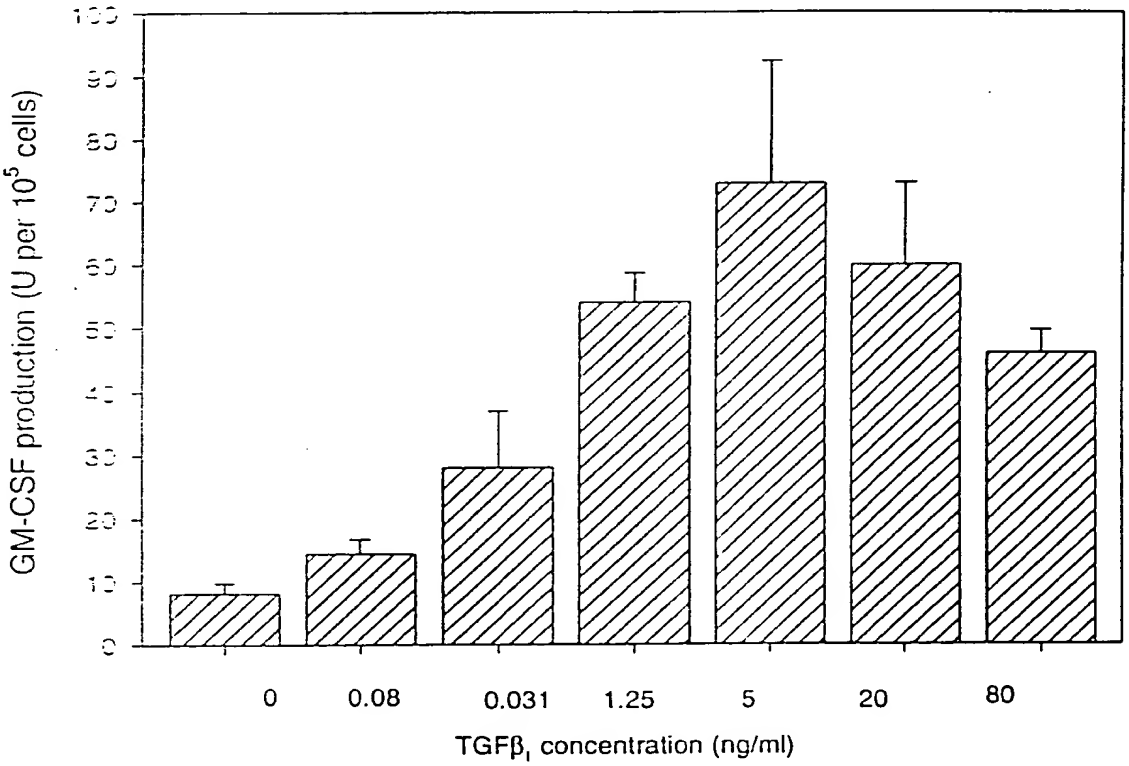
GM-CSF production (U per 10^5 cells)
0
0.08
0.031
1.25
5
20
80
TGFβ1 concentration (ng/ml)

FIGURE 4

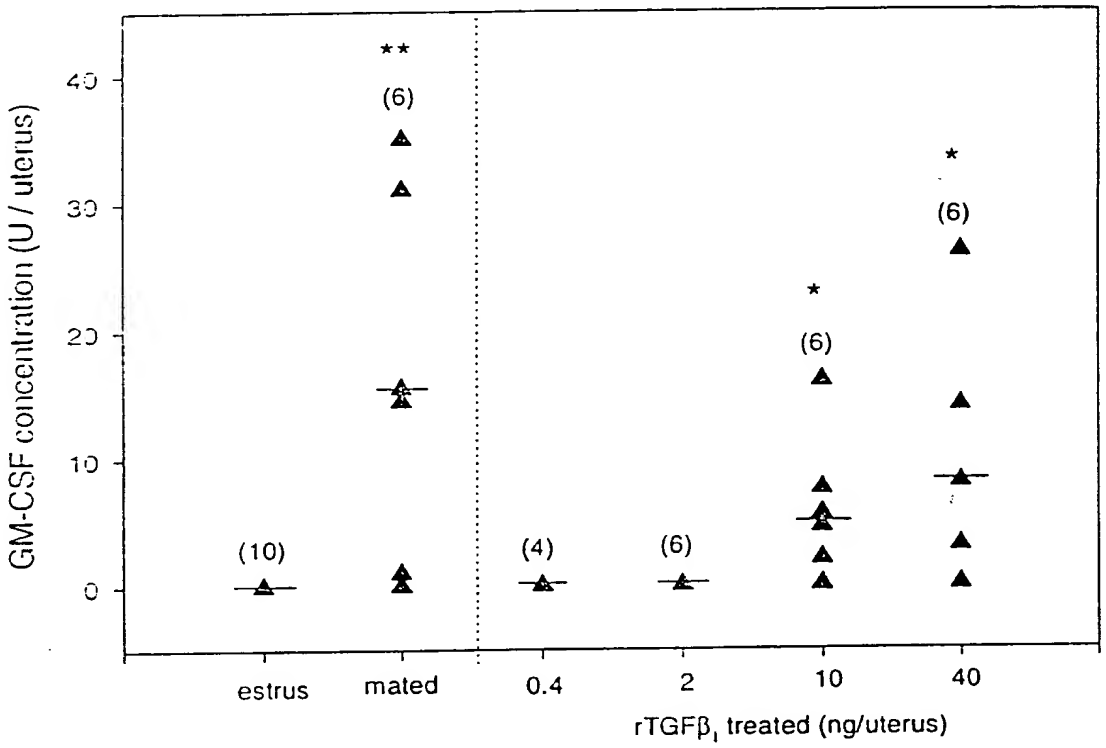
GM-CSF concentration (U / uterus)
40
30
20
10
0
**
(6)
*
(6)
*
(6)
(10)
(4)
(6)
estrus
mated
0.4
2
10
40
rTGFβ1 treated (ng/uterus)

FIGURE 5

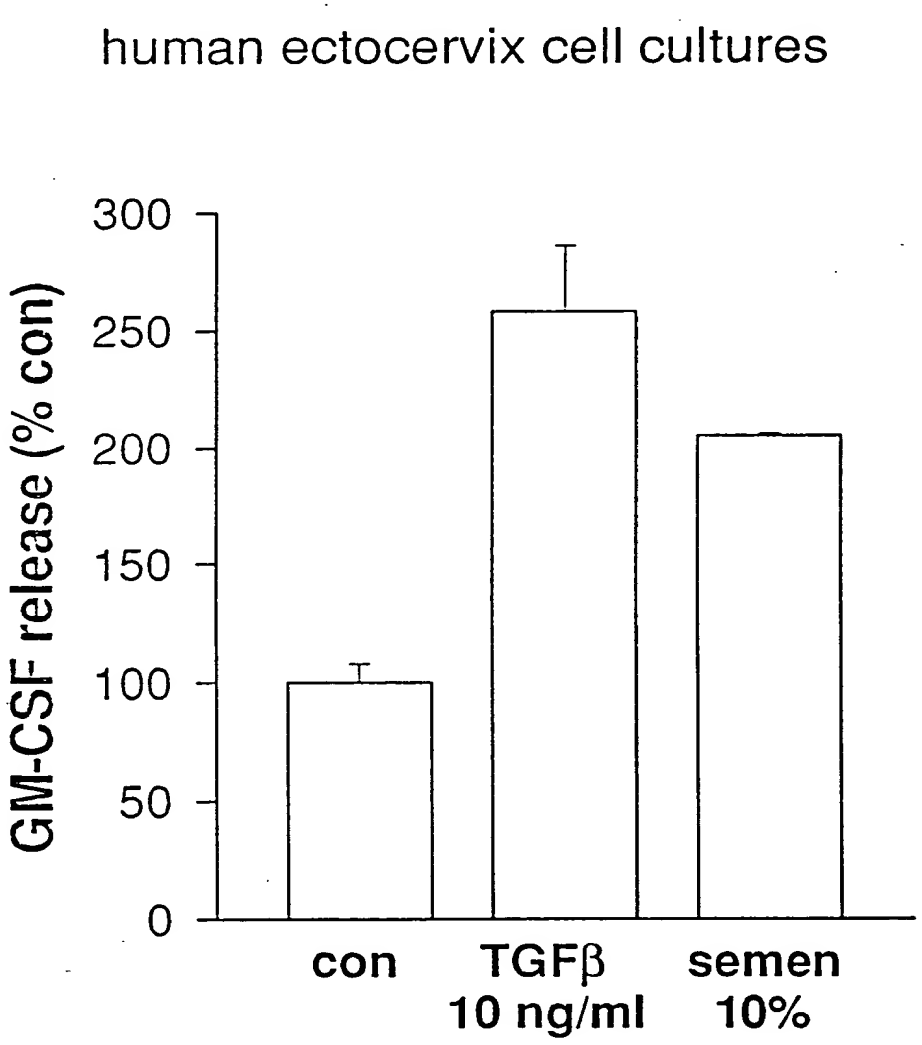
human ectocervix cell cultures
GM-CSF release (% con)
300
250
200
150
100
50
0
con
TGFβ
10 ng/ml
semen
10%

FIGURE 6

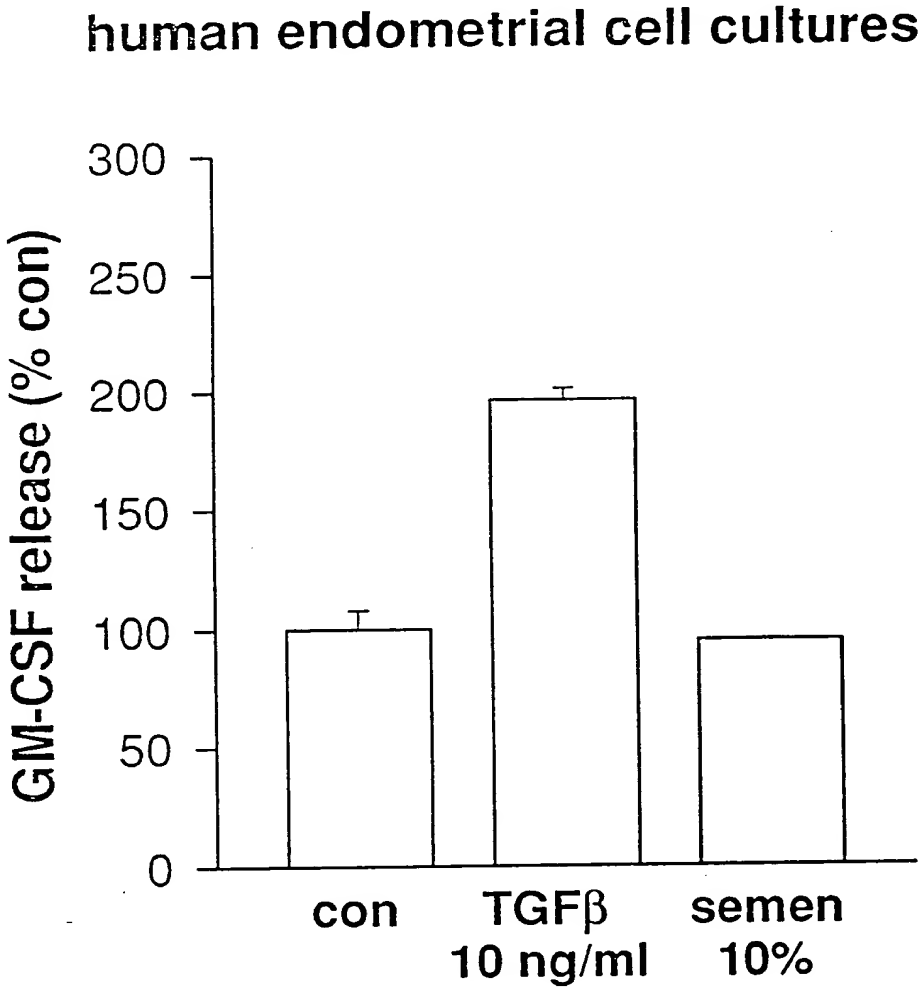
human endometrial cell cultures
GM-CSF release (% con)
300
250
200
150
100
50
0
con
TGFβ
10 ng/ml
semen
10%

FIGURE 7

Antibody treatment	CD45	F 4/ 80	MAC-1	Ia antigen	RB6-6C5	Negative control (eosinophils)
estrus control (Carrier injected) n =5	19.31 (11.91 -25.83)	21.12 (15.49 - 24.36)	17.21 (10.3 - 25.8)	20.49 (13.2 -26.9)	14.4 (8.69 - 19.6)	4.49 (3.63 - 7.0)
da[illegible]mated n= 4	55.92 ** (41.5 - 78.6)	46.0 * (32 - 67.44)	62.58 ** (55.76 - 67.99)	49.9 ** (37.6 - 76.20)	44.87 ** (34.04 -52.4)	13.0 ** (9.72 -19.9)
20 ngm. $rTGF\beta_1$ n=4	41.28 * (23.8 -57.4)	52.87 ** (41.0 - 67.3)	41.30 ** (36.4 - 53.78)	40.12 ** (32.8 - 54.04)	24.55 * (15.2 -38.996)	14.9 ** (11.1 - 19.2)

TABLE 1

The effect of pregnancy and psuedopregnancy on rejection of Balb/c JR-5 fibrosarcoma cells in Balb/k mice.

Female	Male	status at JR-5 injection	tumor growth at day 17 (%)	median tumour size
Balb/c		virgin	11 / 11 (100)	++++
Balb/c	Balb/c	d4 pregnant	5 / 5 (100)	++++
Balb/k		virgin	0 / 10 (0)	-
Balb/k	Balb/c	d4 pregnant	5 / 5 (100)	+++
Balb/k (ut lig)	Balb/c	d4 psuedopregnant	9 / 9 (100)	+++
Balb/k	C57Blk x CBA	d4 pregnant	4 / 8 (50)	+
Balb/k (ut lig)	C57Blk x CBA	d4 psuedopregnant	4 / 8 (50)	+

* Balb/c (H-2d) or Balb/k (H-2d) female mice were mated with Balb/c or C57Blk x CBA F1 (H-2b/k) studs. In some experiments the uteri of Balb/k females were ligated at the oviductal junction 2 weeks prior to mating (ut lig). The day of finding a vaginal plug was designated day 1 of pregnancy or psuedopregnancy. Balb/c tumor cells (JR-5 fibrosarcoma cells, 10^5) were injected s.c. on day 4, and tumor growth (diameter) was measured on day 17 of pregnancy or psuedopregnancy (++++ = > 8 mm; +++ = > 5 mm; + = 1-3 mm).

TABLE 2